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       Novel Selective Antiestrogens for Breast Cancer Prevention
       and Therapy

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13. ABSTRACT (Maximum 200 Words)

We have conceived of an approach to prepare by combinatorial methods, libraries of novel ligands for the estrogen receptor, by the creation of simple amide or five-membered ring heterocyclic core structures that display peripheral substituents (phenols, aliphatic groups, etc.) commonly found in non-steroidal estrogens. These novel estrogens might be useful in the treatment or prevention of breast cancer.

We have made good progress on the preparation of novel estrogen of the diphenyl carboxamide class, the diphenylsulfonamide class, the phenyl benzylcarboxamide and sulfonamide classes, and the pyrazole, oxazole, thiazole, and imidazole classes. Members of some classes have high affinity for the estrogen receptor, and some of them show high binding and potency selectivity for the estrogen receptor subtype alpha and other selectivity for the subtype beta. We have also developed a convenient solid phase synthesis of the pyrazole class, so that we can prepare conveniently and rapidly larger libraries of the members of what appear presently to be the most promising of these classes of novel estrogens.

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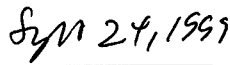
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ANNUAL REPORT: September 1, 1998 – August 31, 1999

PRINCIPAL INVESTIGATOR: John A. Katzenellenbogen

TITLE: Combinatorial Synthesis For The Expedited Discovery Of Novel Selective Antiestrogens For Breast Cancer Prevention And Therapy

ORGANIZATION: University Of Illinois

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INTRODUCTION, GOALS OF THE PROJECT AND APPROACH

[NOTE: For the purpose of continuity, the first three Schemes in the attached Appendix have been taken directly from the original proposal. Schemes, figures, and tables in which new results are presented, are also appended. Compound numbering is somewhat arbitrary, since they are taken from manuscript drafts currently in preparation; the numbering is, however, unambiguous.]

Antiestrogens such as tamoxifen are widely used in the treatment of hormone responsive breast cancer and recently shown to be effective in breast cancer prevention[1-5]. Tamoxifen, however, as well as the pure ICI antiestrogens, are not ideal agents, because they cause vaginal atrophy and menopausal host flashes, induce osteoporosis (pure antiestrogens), and may cause endometrial and liver cancer (tamoxifen)[6-14]. Thus, there is a need for the development of selective antiestrogens with an improved endocrine profile for use in the treatment and prevention of breast cancer[15-16].

Recent advances in our understanding of the molecular pharmacology of estrogens and the development of new selective antiestrogens for menopausal bone maintenance, suggest that new selective antiestrogens of this type can be discovered[16-25]. Up to now, however, this search has not been approached in a systematic fashion[26]. Furthermore, the structures of antiestrogens that have been studied to date are quite complex and their synthesis sufficiently challenging, so as not to be amenable to synthesis by solid-phase combinatorial means. Combinatorial synthesis is the fastest growing new technology in pharmaceutical chemistry, and is proving to be a highly expeditious and promising approach to new drug discovery[27-35].

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In preparing for this project, we analyzed the structures of many selective antiestrogens and found that they possess three common peripheral groups (a phenol, a second aromatic group, and a basic side chain) attached to various core structures. Because the core structure appears to function as a scaffold simply to hold these other appendages together, we then designed six novel core structures that will perform the same scaffold function for the peripheral groups, yet are sufficiently simple that they can be readily prepared by solid-phase combinatorial synthesis.

In this project, we proposed to prepare six novel classes of ligands for the estrogen receptor, based on four functional group so far unexplored in the antiestrogen literature: a carboxamide, a sulfonamide, a pyrazole, and an oxazole (and related thiazole and imidazole). Solution-phase syntheses were first to be developed, and then adapted to solid-phase synthesis using an acid-labile linker attached to the common phenol function. Libraries containing larger numbers of the best of these classes were then to be prepared, and all of these compounds then to be assayed for their binding affinity for the estrogen receptor. The estrogen agonist and antagonist activity of those members with high affinity was then later to be determined in cell transfection and proliferation assays, and those with the most appropriate endocrine activity tested in a uterotrophic assay.

The combination of this novel structural insight leading to the design of new core structures for estrogen receptor ligands that can be readily prepared by combinatorial synthesis, together with a set of simple, but effective assays to establish their hormonal activity, should assist in the discovery of novel selective antiestrogens for the treatment and prevention of breast cancer.

<p style="text-align:center">++++++++++++++++++++++++</p>

BODY

Experimental Approach

A General Structural Description of Selective Estrogen Receptor Ligands Suggests Alternate Core Structures that Can be Prepared by Solid Phase Combinatorial Chemistry Methods

As a class, selective antiestrogens can be envisioned as having four structural components (Schemes 1 and 2 in attached Appendix [taken from the original proposal]): *a core structure* (**A**) onto which are appended three other structural elements, *a phenol* (**B**), *a second aromatic group* (**C**) and *a basic side chain* (**D**). The achievement of an appropriate balance of estrogenic vs antiestrogenic activities in each of these series of selective antiestrogens appears to involve a delicate interaction between these component parts of their structure. Curiously, whereas

<p style="text-align:center">6</p>

components **B**, **C**, and **D** are rather similar in almost all of the selective antiestrogens (cf. Scheme 1, selective antiestrogens), the central core structure **A**, which links together the other three components like a scaffold, is quite variable. This suggests that other core structures could replace this central component in selective antiestrogens.

In the original proposal we proposed to explore new antiestrogens specifically designed to have novel core structures that are readily amenable to solid phase combinatorial synthesis and which may prove to be more tissue selective and efficacious for breast cancer. As explained in Scheme 3 in attached Appendix [taken from the original proposal], we identified three simple structure motifs that are found in ER ligands, around which we designed six new classes of potential ligands for the ER that are based on four new core structures (Scheme 3, right column). We anticipated that these four core structures—a carboxamide, a sulfonamide, a pyrazole, and an oxazole (and a related thiazole and imidazole)—would provide a suitable molecular scaffold for the other three components typically found in selective antiestrogens—the phenol, the second aromatic unit, and the basic side chain—so that these new structural classes would also have selective antiestrogenic activity. The unique feature of these new core structures is that, unlike most antiestrogens develop to date, they can be prepared by combinatorial chemistry means.

The first of the motifs, *motif A is an anti-bibenzyl system*, a structural subunit that is found in the potent estrogen hexestrol, as well as in the antiestrogen hydroxytamoxifen (Scheme 3). The structural analogs of this motif that we planned to explore, the diphenylcarboxamide and diphenylsulfonamide (Classes I and II), are well suited to three-component combinatorial solid phase synthesis methods. *Motif B is the homolog of the bibenzyl motif*, a substructure that is exemplified in the potent estrogen benzestrol and the selective estrogen raloxifene, but is otherwise largely undeveloped. We planned to explore several structural analogs of the homobibenzyl motif that are better suited for solid phase three-component combinatorial synthesis, benzylic homologs of the carboxamides and sulfonamides (Classes III and IV) and pyrazoles (Class V) (see below and Schemes 6 and 7). Finally, *Motif C, a syn-bibenzyl system* found in tamoxifen and centchroman, is the motivation for the three-component combinatorial synthesis of a series of heterocyclic analogs, oxazoles, thiazoles, and imidazoles (Class VI).

As we discuss below, we have made good progress evaluating the synthetic feasibility and ER binding affinity of members of all of these classes, as well as some others.

Results and Discussion for Year 2

The goals in Year 2 of our Statement of Work are listed below; each point will be discussed in the following sections

Year 2

- Complete adaptation of solution phase synthesis to solid phase.
- Isolate and fully characterize representative members produced by solid phase synthesis. Determine yield and characterize impurities.
- Compare estrogen receptor binding of representative members of Class I-VI ligands prepared by solution vs solid phase.
- Begin synthesis of full Class I-VI libraries.
- Begin cell proliferation and cell-based transfection assays.

Complete adaptation of solution phase synthesis to solid phase.

Because they are both the most unusual structurally and have the highest ER binding affinities obtained so far, we have selected the pyrazoles (Class V) as the ER ligands whose synthesis was to be explored by solid phase methods. We have modified the approach shown in the original proposal for solid phase methods to prepare members of Class V, the pyrazoles. Because rather vigorous conditions were required to deprotect the protecting group on the second phenol substituent, we chose to attach the first phenol to the resin directly through a benzyl ether linkage (Merrifield), rather than through the more acid-labile and expensive Wang resin. Thus, the overall scheme for resin attachment [36-37], pyrazole synthesis, and cleavage/deprotection are shown in Scheme 4:

We have spent considerable time working out conditions to ensure that the loading of the resin proceeds in optimal yield, and we have used IR and NMR spectroscopy on polymer-bound material to monitor all of the transformations [38-40]. We can follow the formation of the 1,3-diketone in the Claisen condensation using a ^{1}H NMR-MAS nanoprobe by observing the loss of the methylene proton signal α to the ketone and subsequent appearance of the methine signal at approx. 5 ppm (additional diagnostic chemical shifts are also prevalent). Formation of the pyrazole by reaction of hydrazine with dione can easily be followed by monitoring progressive disappearance of the carbonyl (C=O) band at 1670 cm^{-1}.

Isolate and fully characterize representative members produced by solid phase synthesis, and determine yield and characterize impurities.

We have also worked to develop a consistent and efficient method to isolate the product pyrazoles free from inorganic contaminants and to characterize the level of residual synthetic

precursors that are present in the sample. We developed a novel way to prepare an anionic exchange resin in a carbonate form, completely free from alkali metal cations. We can use this resin in the final work-up of the final pyrazoles freed from the resin. It removes mineral acids and various halide metal ions, and leaves behind only volatile materials (e.g., methyl borate) that are removed upon the final drying of the sample. As a result, we can obtain our organic products free from contamination by inorganic material. The major by-products are the corresponding β-diketone intermediate, and in some cases small amounts of the starting ketone. Despite our attempts to use various ketone scavenging resins (e.g., hydrazide resins), we have not been able to develop a batch-wise method to remove these contaminants. We have demonstrated, however, that even high levels of these ketones do not interfere with our receptor binding assay, and, in any case, they are readily removed by a simple chromatographic steps, see below.

Radial chromatography or steep gradient elution of a reversed-phase HPLC column under a standardized program prove to be robust approaches to characterizing product purity. Because the pyrazoles are uniformly fluorescent, we can use fluorescence detection to identify which of the eluted peaks is due to the pyrazole. In a number of cases we are preparing pyrazoles that have different substituents on positions 3 and 5. In this case, our synthetic approach produces two regioisomers in comparable yields. We can, in most cases, separate these isomers by HPLC, but assigning their structures is difficult. Therefore, at this point, we have tested them separately. We are developing a regioselective synthesis of these isomeric pyrazoles that should enable us to prepare them in an unambiguous fashion. This approach will be reported in the future.

Compare estrogen receptor binding of representative members of Class I-VI ligands prepared by solution vs solid phase.

Originally, we had been concerned that impurities, which might arise from the solid phase synthesis, would interfere with our assays of estrogen receptor binding. We studied this quite carefully with the pyrazoles, but, as mentioned in the section above, by the work-up method we have developed, the only impurities are the starting ketone on the 1,3-diones, and these do not interfere with the binding assay, even at high concentrations. Furthermore, they are removed in the routine purification steps that we use on the final pyrazoles.

In several spot checks of pyrazoles prepared by solid phase methods, we obtained binding affinity values that were equal, within the statistical resolution of our assay (coefficient of variation 0.3) to that we obtained on the material prepared by solution phase methods.

Begin synthesis of full Class I-VI libraries.

As mentioned earlier, we have focused our attention on the pyrazole class, because compounds in this class as so far shown the highest binding affinities for the estrogen receptor. We have prepared two libraries of pyrazoles, a 12-member trial library and a 96-member library.

The diones needed for this (and the larger library) were prepared in a single batch, using a homemade reaction block capable of holding 16 sealed conical polypropylene tubes as reaction vessels that was rotated in an oven at 40 °C for 4 h, using a modified rotary evaporator motor. After washing the resins and drying them overnight, we verified dione formation by nanoprobe ^{1}H NMR•MAS. Each dione resin was then split into the appropriate number of portions and reacted with the appropriate hydrazine, the final products being cleaved/deprotected with BBr_3. For this smaller 12-member library, the cleaved material was collected, treated to a minimal workup (MeOH, passage through a SiO_2 plug), and analyzed for purity by HPLC.

Shown in Table 1 are the HPLC-evaluated purities and the ER binding affinities for the C-(4)-*iso*-butyl pyrazoles in the 12-membered library. The HPLC purity values listed were obtained on the pyrazoles after only minimal workup, yet some of these are quite high (>90%). The pyrazoles derived from *t*-butylhydrazine reacted the most poorly overall. Prior to binding affinity determination, all compounds were purified by radial chromatography, so that their purities were at least 80 %. The molecular ions of all purified pyrazoles in the C(4) *i*-butyl series were also verified by ESI-MS.

The binding affinities of these pyrazoles were determined in a competitive radiometric binding assay, using [^{3}H]estradiol as tracer and lamb uterine cytosol as a source of ER, and they are expressed as relative binding affinity values (RBA), with estradiol having an RBA of 100%.

Overall, the *i*-butyl pyrazoles bind to ER with reasonable affinity, and even within this small set of compound some structure-affinity trends are apparent. Clearly, there is a primary preference for hydroxy substitution at R_1, as in general, the pyrazoles in the **11e-h** series bind better than those in the **11a-d** and **11i-l** series. Bromine substitution (series **11i-l**) is unusual for non-steroidal ligands, but pyrazole **11j** has a reasonable affinity of 3.3%. The high affinity of the *t*-butyl pyrazole **11h** suggests that bulky substituents other than phenyl are tolerated at R_2. The highest affinity members of this small pyrazole library, **11b** and **11f**, contain two and three hydroxyl substituents respectively. The similar but lesser affinity diphenolic pyrazole **11e** suggests that two distinct binding orientations may exist for **11e** and **11b**.

To prepare the 96-member library, we used the Polyfiltronic's 96-well Unifilter® plate. This system was specifically designed for combinatorial chemistry in a standard 96-well format, and it was the first of its kind available for a modest cost (ca. $2000). The Unifilter® plate is constructed of glass-embedded polypropylene, and it has a single underlying membrane, which is

fused to the bottom each well. The membrane is designed to hold back most organic solvents, except when a vacuum is applied. Unfortunately, the Polyfiltronics plate did not withstand our conditions for pyrazole formation using prolonged heating with toluene at 80 °C. However, by systematic variation of reaction conditions we found new conditions that did not affect the plate material and were generally satisfactory for pyrazole formation. Shown below in Scheme 4 is the overall optimized solid phase synthesis route to the 96-member pyrazole library. The notable modifications from our original route are the conditions for the pyrazole-forming step and final workup procedure involving the bicarbonate resin **16b**.

The individual components chosen for the 96-member library are shown in Scheme 5. The components used to prepare the *i*-butyl library from Table 1 were again included as standards to measure the success and reproducibility of the synthesis and to verify the RBA assays. The progress of pyrazole formation for suspected "worst-case" combinations, such as CF_3-phenyl and *t*-butyl hydrazines reacting with halogen substituted diones was monitored using FT-IR. The disappearance of the C=O signal at 1670 cm^{-1} was a reliable marker for determining the progress of individual reactions. Unfortunately, for several CF_3-substituted hydrazines we were unable to drive the reaction to completion, even after subjecting these resins to fresh reagent and heating for an additional 20h. In any event, cleavage/deprotection with BBr3 followed, and reactions were carefully quenched with MeOH and then incubated with bicarbonate resin **16b** for 1h at 50-60 °C to ensure complete HBr neutralization and Br-ion sequestration. Upon cooling, the pyrazole products were collected and concentrated, then reconstituted in 1 mL MeOH and analyzed using a standard, steep gradient reversed phase high-throughput HPLC column.

The HPLC purities for the final pyrazoles are shown in Figure 1, according to gray-scaled ranges. The average purity for the library was 50% (±15%). This is not an unreasonable level of purity, when you consider that this library included components, such as *t*-butyl hydrazine hydrochloride, which we have found to be less reactive than the aromatic hydrazines. As before, the principal impurities could be identified as the unreacted dione precursors, which we had previously shown did not affect the results of the binding assay. Based on product yields, *p*-CF3-substituted phenyl hydrazines appear to be even less reactive than *t*-butyl hydrazine hydrochloride, as this group of pyrazoles had the lowest overall purity of the whole library.

Shown in Figure 2 are the RBA values for the 96-member library, indicated as ranges according to the gray-scale legend. Several members of the library showed appreciable affinity for the ER. Particularly gratifying was the fact that most members of the 12-member *i*-butyl control library (Table 1) had RBA values which were generally reproduced quite well in the larger library (±30% relative deviation), with the exception of **11f** (7.6% vs. 23%), for which the determination in the original 12-member library was later shown to be low. The binding affinity

of sixteen additional select members was also re-tested after chromatographic purification (>80%), and the RBA values for these members also agreed quite well with the original determinations.

The use of an affinity array chart in Figure 2 permits a rapid, visual assessment of binding affinity patterns. For example, it is readily apparent that for pyrazoles in both the R_1 ethyl and i-butyl series (column 2 and 8), those with HO-substituents at R_2 have overall, the highest affinity. Within these two series (columns 2 and 8), a number of substituents are tolerated at R_3, the best being p-HO-C_6H_4, for which the two highest affinity pyrazoles are represented, pyrazole **B-2** (14%) and **B-8** (23%). For pyrazoles with $R_3 = p$-HO-C_6H_4 (row B), a number of substituents are tolerated to varying degrees; those with fluorine substituents on R_2 for both the ethyl and i-butyl series bind moderately well (RBA = 1-5 %). For members with $R_1 = i$-butyl and $R_3 = p$-HO-C_6H_4, even more polar substituents appear to bind well, particularly noteworthy is the m-HO analog **B-9** which has a relative binding affinity of 6.8% and the fluoro-derivative **B-11**. By contrast, few other R_3 substituents are well tolerated by the ER, one exception, however, being the t-butyl pyrazoles **H-2** and **H-8**, which each have affinities of 6-7%. As was the case with pyrazole **11j** from the 12-member i-butyl library, several pyrazoles with p-bromo-substituents at R_2 have reasonable affinity (~ 2-3%); however, these affinities are much lower when $R_1 =$ ethyl (RBA <1 %).

Other more subtle trends can be found upon closer examination, some of which are illustrated in Figure 3. For example, in both the ethyl and i-butyl series, m-CH_3-substituents on the N-phenyl group (R_3) are tolerated 3-fold better than p-CH_3-substituents, but overall affinity is greater when $R_1 = i$-butyl (**C-2, C-8** vs. **D-2, D-8**). The fact that the $meta$-CH_3 isomers bind better than the $para$-CH_3 isomers suggests that there is more limited room for substituent extension in this region of the receptor-binding pocket.

Additional significant $meta$ and $para$-methyl comparisons cam be made between the m-$CH_3C_6H_4$-ethyl and i-butyl members **D-2** and **D-8** relative to the protio-pyrazoles **A-2** and **A-8** (Figure 3). Interestingly, adding a m-CH_3 group to the R_3 substituent to the R_1 i-butyl pyrazoles results in a slight increase in affinity (**A-8** to **D-8**), whereas the same substitution decreases affinity 4-fold in the related ethyl congeners **A-2** and **D-2**. A similar trend reversal is observed between the protio-pyrazoles, and the trihydroxy-pyrazoles **B-2** and **B-8**. Thus, substitution of ethyl for i-butyl lowers the affinity 4 fold for the N-phenyl members; in contrast, the same change causes a slight increase in the trihydroxy-pyrazoles. The interesting changes observed for the ethyl and i-butyl-substituted pyrazoles suggest that more than one binding mode may be operative, or conversely that a single binding orientation exists, but significant changes in more than one subpocket is occurring between the ethyl and i-butyl series.

Begin cell proliferation and cell-based transfection assays.

We just begin to do cell-based transcription assays on the pyrazoles that we have prepared, as well as some of the amide compounds that we have reported on earlier. These are shown in the figures below. From the results in Figure 4, it is clear that some of the pyrazoles, notably compounds **34** and **36**, have very high potency selectivity for the estrogen receptor subtype alpha. From the results in Figure 5, it is evident that one of the amide compounds has significant potency selectivity for estrogen receptor subtype beta.

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KEY RESEARCH OUTCOMES

Progress in Relation to the Statement of Work

The complete three year Statement of Work, presented in the original proposal of July 1996, is shown below:

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ORIGINAL STATEMENT OF WORK

Project Period: July 1, 1997– June 30, 2000 (3 years)

Year 1
- Synthesis of representative members of Class I-VI ligands by solution phase methods.
- Isolate, purify, and fully characterize these members.
- Measure estrogen receptor binding affinity of representative members of Class I-VI ligands
- Begin to adapt solution phase syntheses to solid phase.

Year 2
- Complete adaptation of solution phase synthesis to solid phase.
- Isolate and fully characterize representative members produced by solid phase synthesis. Determine yield and characterize impurities.
- Compare estrogen receptor binding of representative members of Class I-VI ligands prepared by solution vs solid phase.
- Begin synthesis of full Class I-VI libraries.
- Begin cell proliferation and cell-based transfection assays.

Year 3

- Complete synthesis of full Class I-VI libraries.
- Complete estrogen receptor assay of full libraries at two concentrations.
- Reassay the members with detectable estrogen receptor binding affinity by quantitative titration assay.
- Assay the members with high estrogen receptor binding affinity in the cell proliferation and cell-based transfection assays.
- Assay uterotrophic activity of the most promising members in rats.

===

It is evident from the results presented in the preceding sections that we have fulfilled all of the components of the Statement of Work for Year 1. In addition, we have made progress on all of the elements of the Statement of Work for Year 2. The only modification that we have made is that in the development of larger libraries by solid phase methods, we have focused on a single class of novel estrogens, the pyrazoles, because the members of this class appear to be the most promising, in terms of their affinity for the estrogen receptor and the degree of estrogen subtype selectivity that we can see. We are beginning to make progess on the other work elements for Year 3.

The key outcomes for the second year of this project are:
- Complete adaptation of solution phase synthesis to solid phase.
- Isolation and full characterization of representative members produced by solid phase synthesis, determining yield and purity, and characterizing impurities.
- Comparison of estrogen receptor binding of representative members of Class I-VI ligands prepared by solution vs solid phase.
- Preparation of two libraries, using solid phase synthesis, of pyrazoles.
- Begin cell-based transfection assays to establish potency and estrogen receptor subtype selectivity.

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REPORTABLE OUTCOMES

- Five manuscripts are currently in preparation that cover the work that has been done on this project up to now.
- Presentations have been made at two American Chemical Society meeting by the PI and the principal co-worker, Shaun Stauffer. Numerous seminars given by the PI have included work developed under this project.
- A provisional patent has been submitted to the US Patent Office covering the 5-membered ring heterocyclic estrogens. A full patent application is being prepared.
- Shaun Stauffer, my principal co-worker on this project, completed his Ph.D. degree in the fall of 1999.
- Shaun Stauffer, my principal co-worker on this project, is currently a postdoctoral fellow with Professor John Hartwig at Yale University, supported by a postdoctoral fellowship from the National Institutes of Health. The topic of Shaun's NIH postdoctoral fellowship application derived from the experience he developed working on this project.

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CONCLUSIONS

From the results that we have achieved so far, we have identified several new series of non-steroidal estrogens and we have adapted the currently most promising of these to a combinatorial synthesis approach on solid phase. Some of the members of these series also have high affinity for the estrogen receptor and show affinity and potency selectivity for the two estrogen receptor subtype. Thus, the project is progressing well along the lines that were originally envisioned in the initial proposal.

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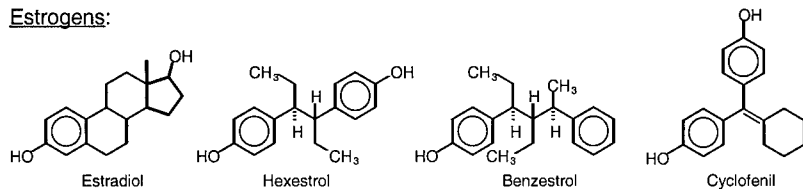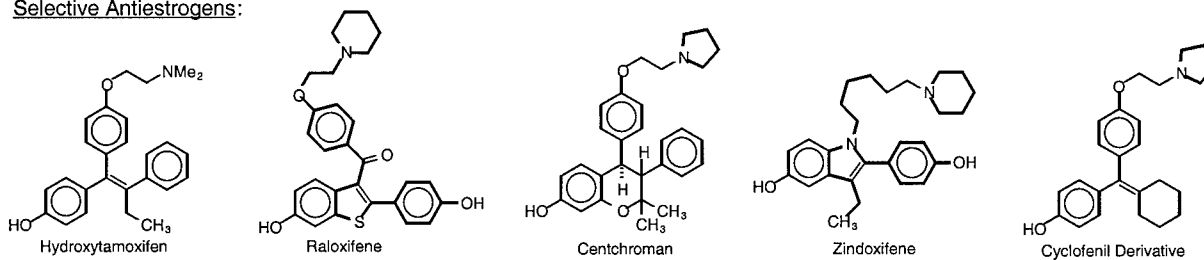
APPENDIX

Table 1. HPLC Purity Determination and Estrogen Receptor Binding Affinities (RBA Values) of C(4) i-Butyl Pyrazole Library.

Compound	R_1	R_2	HPLC purity[a]	% RBA[b]
11a	H	Ph	80 %	0.38 / 0.13[c]
11b	H	p-OHC$_6$H$_4$	67 %	9.3 / 0.86[c]
11c	H	p-FC$_6$H$_4$	92 %	0.04
11d	H	t-Bu	29 %	0.47
11e	OH	Ph	62 %	5.4
11f	OH	p-OHC$_6$H$_4$	>99 %	7.6
11g	OH	p-FC$_6$H$_4$	90 %	4.0
11h	OH	t-Bu	23 %	3.8
11i	Br	Ph	75 %	0.76 / 0.01[c]
11j	Br	p-OHC$_6$H$_4$	> 85 %	3.3
11k	Br	p-FC$_6$H$_4$	> 80 %	0.17
11l	Br	t-Bu	28 %	0.90

[a]RP-HPLC, 80% MeOH:H$_2$O, flow rate of 1.0 mL/min, detection at 254 nm (performed before purification by radial chromatography). [b]Relative Binding Affinity (RBA) values determined by a modification of our standard competitive binding assay using only three concentrations of ligand; all compounds tested were at >80% purity. [c]Independent assays performed on the two individual regioisomers.

Scheme 1. Estrogens, Pure Antiestrogens, and Selective Antiestrogens – From the Original Proposal

Estrogens:

Estradiol   Hexestrol   Benzestrol   Cyclofenil

Pure Antiestrogens:

ICI 182,780   RU 54,876

Selective Antiestrogens:

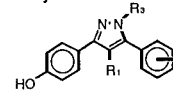Hydroxytamoxifen   Raloxifene   Centchroman   Zindoxifene   Cyclofenil Derivative
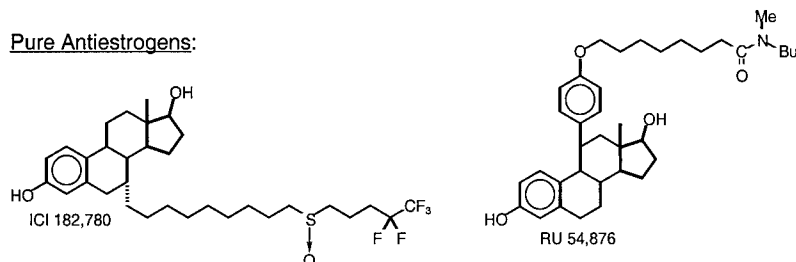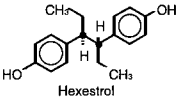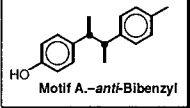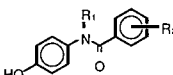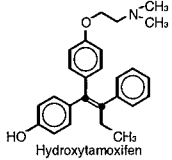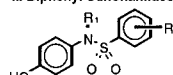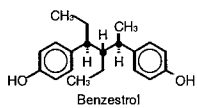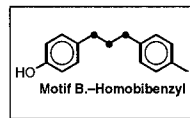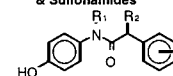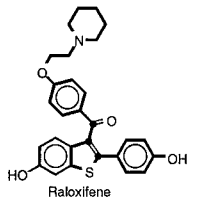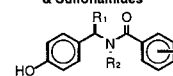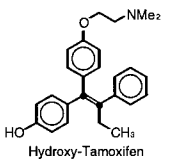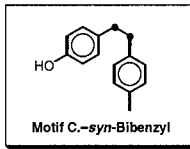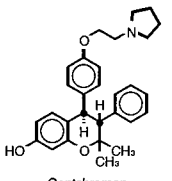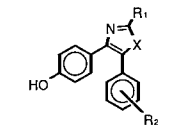
Scheme 2. Structural Components of Selective Antiestrogens
– From the Original Proposal

Basic Side Chain **D**

Core Structure **A**

Phenol **B**

Second Aromatic Unit **C**
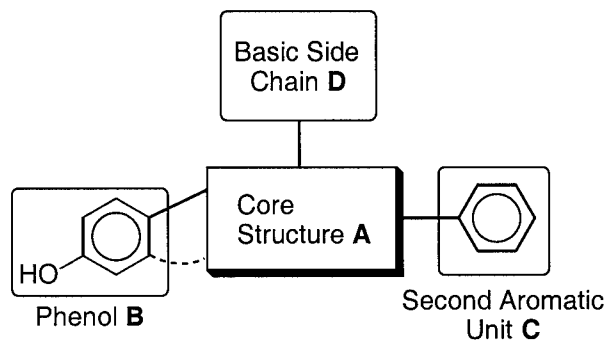
**Scheme 3. Structural Motifs for Estrogen Receptor Ligands
and Their Combinatorial Analogs – From the Original Proposal**

Estrogen Receptor Ligand	Structural Motif	Combinatorial Analog (Class)

Hexestrol

Motif A.–*anti*-Bibenzyl

I. Diphenyl Carboxamides

Hydroxytamoxifen

II. Diphenyl Sulfonamides

Benzestrol

Motif B.–Homobibenzyl

III. Phenyl Benzylcarboxamides & Sulfonamides

(or SO$_2$ in place of CO)

Raloxifene

IV. Benzyl Phenylcarboxamides & Sulfonamides

(or SO$_2$ in place of CO)

V. Pyrazoles

Hydroxy-Tamoxifen

Motif C.–*syn*-Bibenzyl

VI. Oxazoles, Thiazoles, and Imidazoles

(X = O, S, NR)

Centchroman
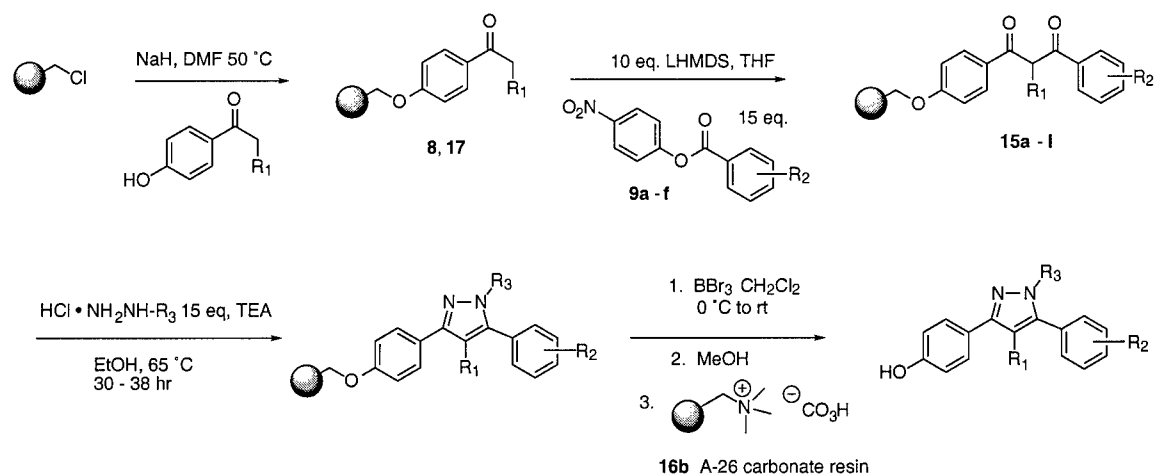
Scheme 4. Optimized route to prepare 96-member pyrazole library for the ER.

Scheme 5. Components for 96-member pyrazole library
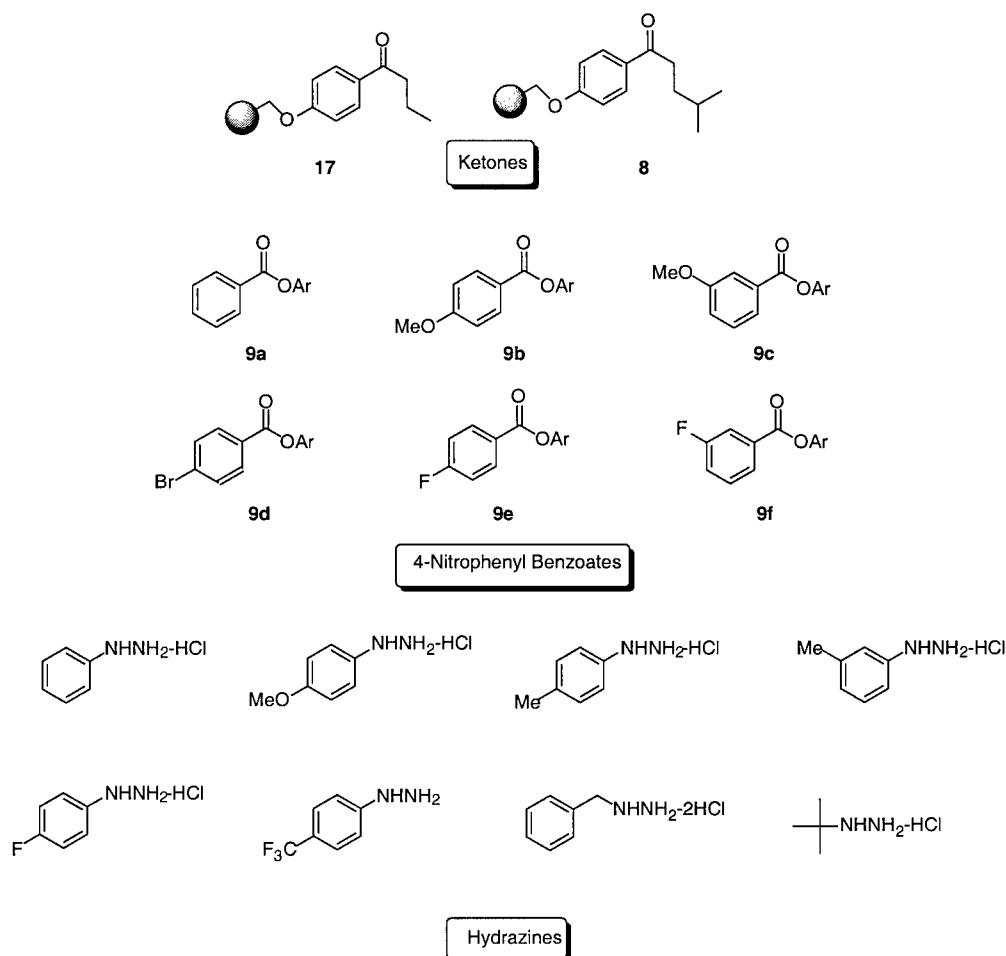
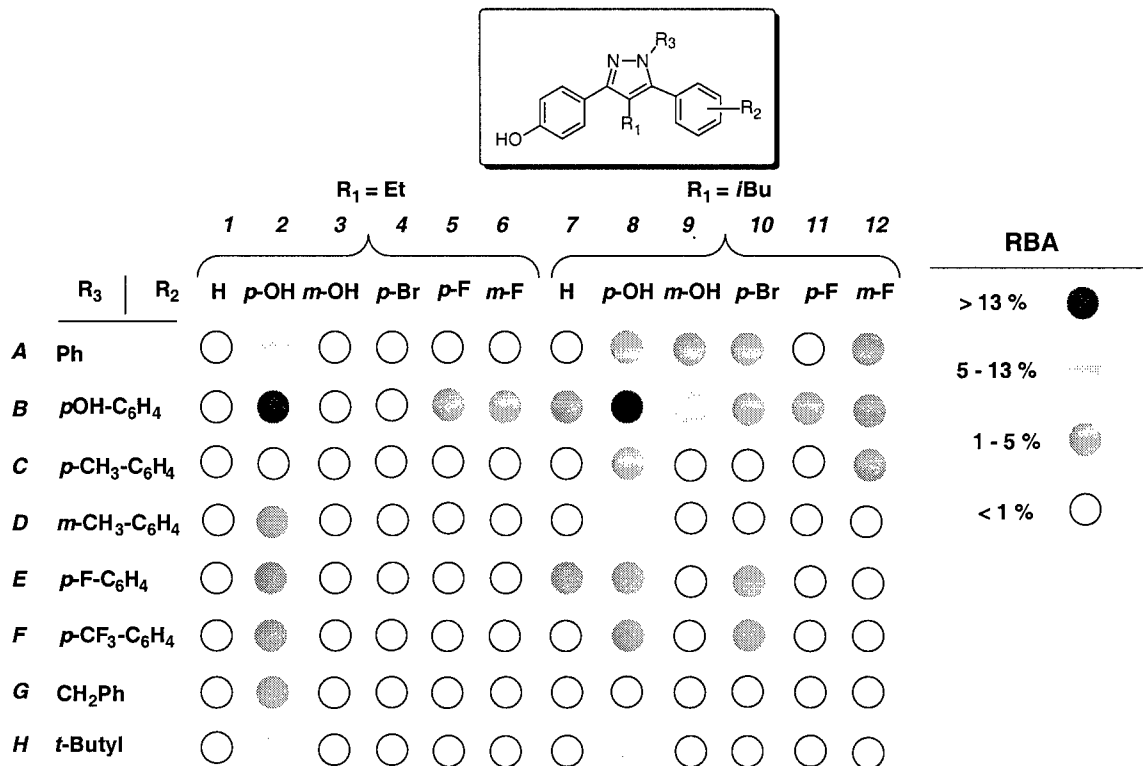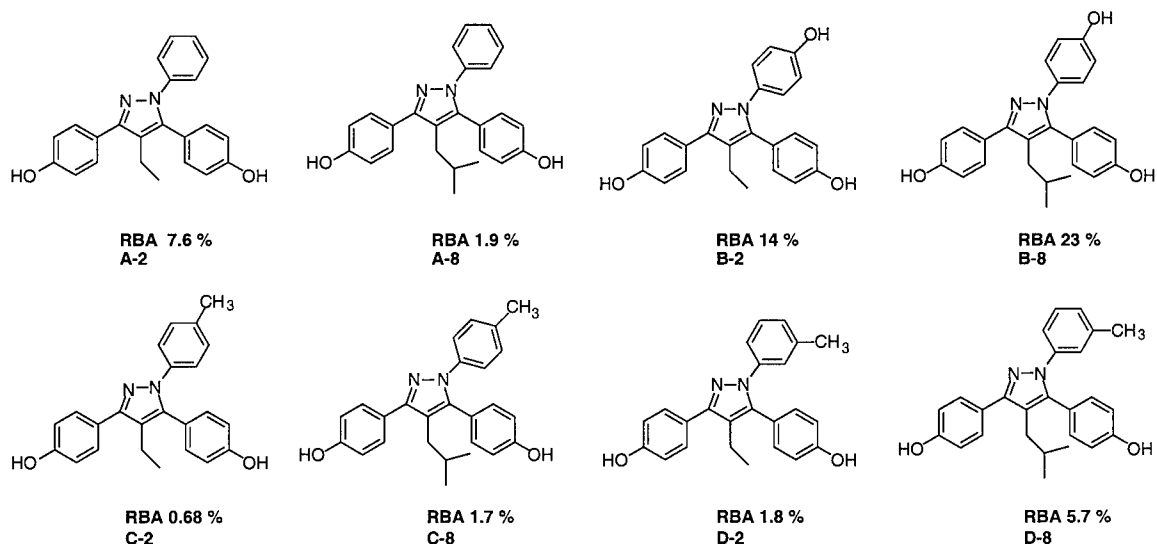**Figure 1.** HPLC purities for 96-member ER library

Figure 2. ER Binding results for 96-member pyrazole library (ave. detected regioisomer ratio 1.4 to 1)

		R_1 = Et						R_1 = iBu					
		1	2	3	4	5	6	7	8	9	10	11	12
R_3	R_2	H	p-OH	m-OH	p-Br	p-F	m-F	H	p-OH	m-OH	p-Br	p-F	m-F
A	Ph												
B	pOH-C_6H_4												
C	p-CH_3-C_6H_4												
D	m-CH_3-C_6H_4												
E	p-F-C_6H_4												
F	p-CF_3-C_6H_4												
G	CH_2Ph												
H	t-Butyl												

RBA
> 13 % ●
5 - 13 %
1 - 5 %
< 1 % ○

Figure 3. Select RBA comparisons between Ethyl and i-Butyl Pyrazoles.

RBA 7.6 %
A-2

RBA 1.9 %
A-8

RBA 14 %
B-2

RBA 23 %
B-8

RBA 0.68 %
C-2

RBA 1.7 %
C-8

RBA 1.8 %
D-2

RBA 5.7 %
D-8

Figure 4. Transcription activation by ERα and ERβ in response to pyrazoles **34** and **36**. Human endometrial cancer (HEC-1) cells were transfected with expression vectors for ERα (solid lines) or ERβ (dashed lines) and an $(ERE)_3$-pS2-CAT reporter gene and were treated with indicated concentrations of ligand for 24h. CAT activity was normalized for β-galactosidase activity from an internal control plasmid. Values are expressed as a percent of the ERα or ERβ response with 1 nM E_2.
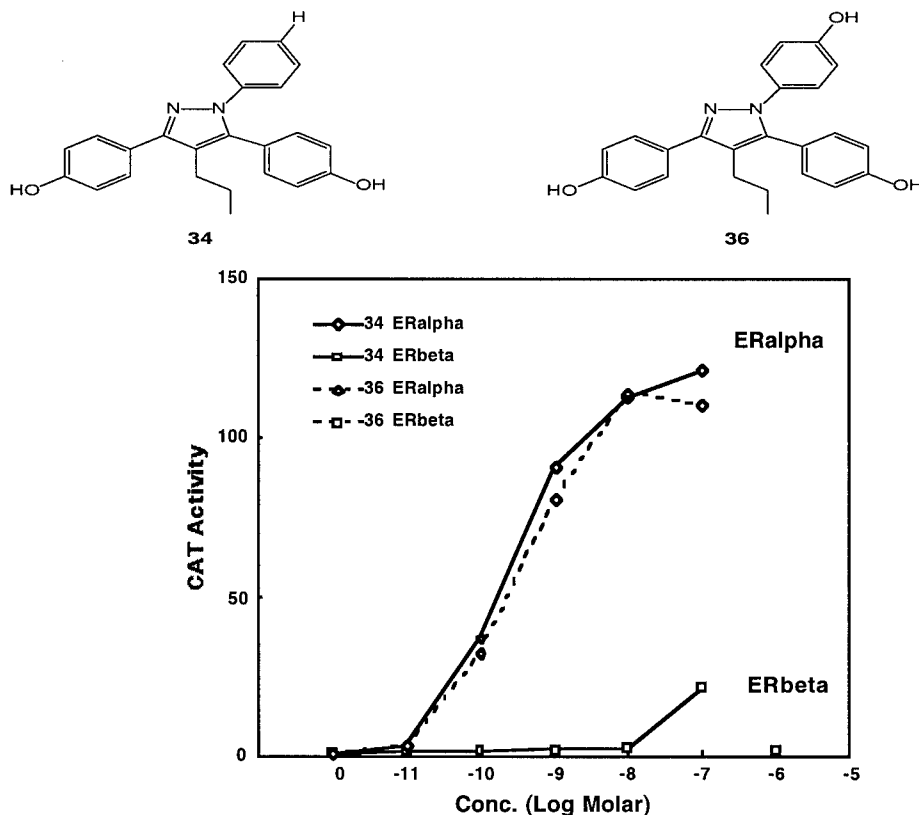
Figure 5. Transcription activation by ERα and ERβ in response to benzamide **16g**. Human endometrial cancer (HEC-1) cells were transfected with expression vectors for ERα (Panel A) or ERβ (Panel B) and an (ERE)3-pS2-CAT reporter gene and were treated with indicated concentrations of E2 or benzamide **16g** for 24h. CAT activity was normalized for β-galactosidase activity from an internal control plasmid. Values are the mean ± SD for three or more separate experiments, and are expressed as a percent of the ERα or ERβ response with 1 nM E2.
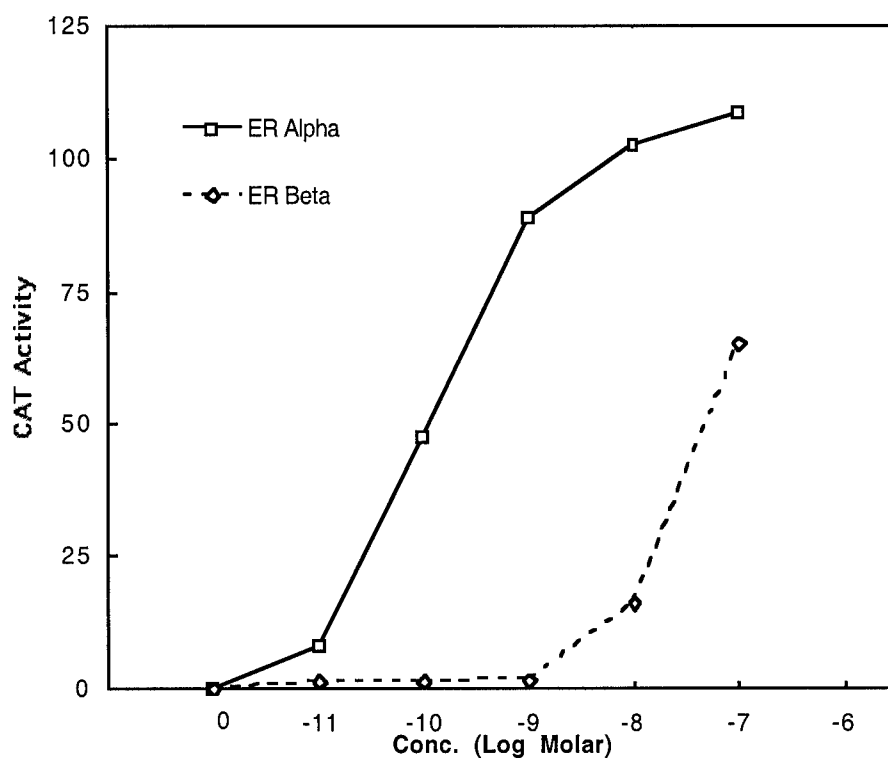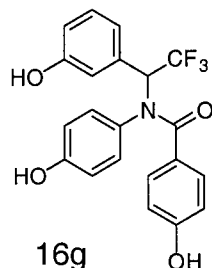
16g

REPLY TO
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